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(21) International Application Number: PCT/US97/03444 (22) International Filing Date: 6 March 1997 (06.03.97) (30) Priority Data: 60/012,916 6 March 1996 (06.03.96) US (71) Applicant *(for all designated States except US)*: RUTGERS UNIVERSITY [US/US]; ASB Annex 2, Bevier Road, P.O. Box 1179, Piscataway, NJ 08855 (US). (72) Inventors; and (75) Inventors/Applicants *(for US only)*: MALIGA, Pal [US/US]; 72 Yorktown Road, East Brunswick, NJ 08815 (US). SIKDAR, Samir, R. [IN/IN]; 711 Nawabbagan BO Chinsurah, District Hooghly, West Bengal-712101 (IN). REDDY, Siva, Vanga [IN/IN]; B1/1160 Varsant Kunj, New Delhi 70-110070 (IN). (74) Agents: HAGAN, Patrick, J. et al.; Dann, Dorfman, Herrell and Skillman, Suite 720, 1601 Market Street, Philadelphia, PA 19102 (US).	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). **Published** *With international search report.* *Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: PLASTID TRANSFORMATION IN ARABIDOPSIS THALIANA

(57) Abstract

    This invention provides methods and compositions for obtaining transplastomic *Arabidopsis* and *Brassica* plants. Specifically, the method provides culturing protocols and compositions that facilitate the regeneration of transformed plants following delivery of exogenous, beneficial DNA molecules.

PLASTID TRANSFORMATION IN *ARABIDOPSIS THALIANA*

FIELD OF THE INVENTION

The present invention relates to the field of
transgenic plants. Specifically, the invention provides
10   compositions and methods for the transformation of
plastids in plants from the *Cruciferae* family.

BACKGROUND OF THE INVENTION

Several publications are parenthetically
15   referenced in this application in order to more fully
describe the state of the art to which this invention
pertains. Full citations for these references are found
at the end of the specification. The disclosure of each
of these publications is incorporated by reference in the
20   present specification as though set forth herein in full.

The plastid genome of higher plants is a
circular double-stranded DNA molecule of 120-160 kb which
may be present in 1,900-50,000 copies per leaf cell
(Palmer, 1991; Bendich, 1987). Stable transformation of
25   the tobacco plastid genome (plastome) has been achieved
through the following steps: (i) introduction of
transforming DNA, encoding antibiotic resistance, by the
biolistic process (Svab et al. 1990a; Svab and Maliga
1993) or PEG treatment (Golds et al. 1993; O'Neill et
30   al., 1993), (ii) integration of the transforming DNA by
two homologous recombination events and (iii) selective
elimination of the wild-type genome copies during
repeated cell divisions on a selective medium.
Spectinomycin resistance has been used as a selective
35   marker encoded either in mutant plastid 16S ribosomal RNA
genes (Svab et al. 1990a; Staub and Maliga 1992; Golds et

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al. 1993), or conferred by the expression of an
engineered bacterial *aadA* gene (Svab and Maliga 1993).
Vectors which utilize *aadA* as a selectable marker gene
and target the insertion of chimeric genes into the

5   repeated region of tobacco plastid genome are available
(Zoubenko et al., 1994). Selection of plastid
transformants by kanamycin resistance, based on the
expression of neomycin phosphotransferase (*kan* gene), is
more difficult but also feasible (Carrer et al., 1993;

10   Carrer and Maliga, 1995).

To date, stable plastid transformation in
higher plants has been reported in tobacco only (reviewed
in Maliga, 1993; Maliga et al., 1993). Transplastomic
plants from other agriculturally and pharmaceutically

15   important species are highly desirable. Expression of
foreign genes of interest in the plastids of higher
plants in the family *Cruciferae* provides several
advantages over nuclear expression of foreign genes.
These are 1) expression of exogenous DNA sequences in

20   plastids eliminates the possibility of pollen
transmission of transforming DNA; 2) high levels of
protein expression are attainable; 3) the simultaneous
expression of multiple genes as a polycistronic unit is
feasible and 4) positional effects and gene silencing

25   which may result following nuclear transformation are
also eliminated.

SUMMARY OF THE INVENTION

The present invention provides improved methods for

30   the generation of stably transformed, transplastomic
plants. In one embodiment of the invention, cotyledon
cells are cultured in high auxin liquid medium for a
sufficient time period to stimulate uniform cell
division. Intitial culture is at a high density (50-200

35   cotyledons/ 20 ml). The cotyledons are then transferred
to agar-solidified medium for delivery of exogenous,
transforming DNA. Following delivery of transforming

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DNA, the cotyledons are transferred at a lower density
(25-30/50 ml) to a medium containing high cytokinin
levels and the selection agent to facilitate selection of
transformants and plant regeneration. Presence of the
5   exogenous DNA in the plastid genome is then confirmed by
Southern blot analysis or PCR.

The transforming DNA molecules of the invention have
several distinct features. These are 1) targeting
segments flanking the foreign gene of interest consisting
10   of plastid DNA sequences from the plant to be
transformed, thereby facilitating homologous
recombination of the transforming DNA into a pre-
determined region of the plastid genome; 2) a selectable
marker gene disposed within the targeting segment,
15   conferring resistance to a selection agent; 3) 5' and 3'
regulatory sequences derived from plastid DNA operably
linked to sequences encoding a foreign gene of interest
thereby enhancing expression of the transforming DNA and
stability of encoded mRNA; and 4) at least one cloning
20   site adjacent to the selectable marker gene for insertion
of the foreign gene of interest which by itself is not
selectable. Since the selectable marker gene and the
foreign gene of interest form a heterologous block of
contiguous sequence, integration of both genes into the
25   plasid genome is effected.

In another embodiment of the invention, leaf cells
are initially treated with high auxin media, followed by
transformation with the transforming DNA and culturing in
the presence of high cytokinin levels and a predetermined
30   selection agent. Cells containing transformed plastids
are maintained in the presence of the selection agent
facilitating the obtention of homoplasmic cells which can
then be regenerated into transplastomic plants.

Thus, the present invention provides novel methods
35   and compositions for creating transplastomic plants. The
genus *Arabidopsis* belongs to the mustard or crucifer
family (*Brassicaceae* or *Cruciferae*), a widely distributed

family of approximately 340 genera and 3350 species. The
family is of significant economic importance as a source
of vegetable crops, oil seeds, spices and, to a lesser
extent, ornamentals. Much of its agricultural importance
derives from the genus *Brassica*. Examples for *Brassica*
ssp. of economic importance are: *Brassica napus* (oil
seed), *Brassica juncea* (oil seed), *Brassica campestris*
(oil seed), *Brassica juncea* (oil seed), *Brassica*
oleracea (broccoli, cauliflower, cabbage) *Brassica nigra*
(black mustard) and *Brassica hirta* (white mustard).

Plastid transformation in *Arabidopsis thaliana* a
model species for plant research (Meyerowitz and
Sommerville, 1994) and *Brassica ssp.*, an important
agricultural crop is exemplified herein. These methods
are suitable for transformation of plastids in other
plants from the *Cruciferae* family.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing illustrating the
integration of aadA into the Arabidopsis plastid genome
(ptDNA) after transformation with plasmid pGS31A. Figure
1A shows a map of the transformation vector pGS31A, the
ptDNA region containing the integrated spectinomcycin
resistance (aadA) gene (T-ptDNA) and the cognate region
of the wild-type ptDNA. 16SrDNA, rps12/7 and trnV are
plastid genes (Shinozaki et al., 1986). Figure 1B shows
the regions of ptDNA contained in the P1 and P2 probes.
Figure 1C is an autoradiogram showing the results of
Southern blot hybridization confirming integration of
aadA in the plastid genome. The P1 targeting sequences
hybridize to a 2.72-kb fragment in the wild-type (At)
plants and to a larger, 3.82-kb fragment in the
transplastomic line (At-pGS31A-16). Note absence of
wild-type fragment in transplastomic line. The aadA
probe, P2, hybridizes only to the larger transplastomic
fragment.

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Figure 2 is a schematic diagram of the plastid transformation protocol used for *Arabidopsis* leaves.

Figure 3 is a schematic diagram of the different protocols used for obtaining fertile *Arabidopsis* plants from cotyledonary explants of *Arabidopsis thaliana* (RLD) having transformed plastids.

Figure 4 is a map of the plastid targeting region of pGS7 and pGS85A plasmids. Note unique HincII cloning site in plasmid pGS7 and KpnI restriction site in plasmid pGS85, and chimeric kan kanamycin resistance gene. The plastid genes trnV, 16SrDNA and rps12/7 are described in Shinozaki et al., 1986. Site and direction of transcription initiation is indicated by horizontal arrow.

Figure 5 is a sequence of the targeting region of plasmid pGS7. The genes conferring resistance to kanamycin or spectinomycin will be inserted into the marked Hinc II site .

Figure 6 is a sequence of the plastid targeting region of plasmid pGS31A.

Figure 7 is the sequence of the plastid targeting region of plasmid pGS85A.

DETAILED DESCRIPTION OF THE INVENTION

Insofar as it is known, plastid transformation has been demonstrated in tobacco only. A protocol for the transformation of plastids in *Arabidopsis thaliana* and *Brassica napus* has now been developed and the methods utilized to create these transformants are set forth below. The use of *Arabidopsis* and *Brassica* in the following examples is meant to be illustrative of the methods of the invention. The methods disclosed herein

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may be adapted to other plants in the *Cruciferae* family.

The plastids of *Arabidopsis thaliana* have been transformed following biolistic delivery of transforming DNA into leaf cells on the surface of microscopic (1 μm) tungsten particles as described below in Example I. The transforming plasmid pGS31A, used for these experiments carries a spectinomycin resistance (*aadA*) gene flanked by plastid DNA sequences to target its insertion between trnV and the rps 12/7 operon. Integration of *aadA* by two homologous recombination events via the flanking ptDNA sequences and selective amplification of the transplastomes on spectinomycin medium yielded spectinomycin resistant cell lines. Regenerated plants were homoplasmic in that the plastid genome copies had been uniformly altered by the transforming DNA. The efficiency of plastid transformation was low, two in 201 bombarded leaf samples. However, none of the 98 plants regenerated from the two lines were fertile.

These fertility problems were likely attributable to extended periods of treatment with 2,4-D, an auxin (Van der Graaff and Hooykas, 1996). It is possible that shortening exposure time to this agent may overcome the fertility problem. The relatively long growth period of Arabidopsis thaliana to provide a suitable source of leaves for transformation also makes leaves a less desirable tissue source.

Cotyledons and leaves each contain an abundant number of plastid genome copies per cell. Additionally, cotyledons provide a more available tissue source. Accordingly, cotyledon cells have been utilized as recipients for transforming DNA as set forth in Example II below. Cotyledon cells are preferred over leaf cells for practicing the methods of the present invention due to the relatively short (7 days) culturing period to prepare the cells for bombardment with transforming DNA. Another advantage to using cotyledon cells as the target cell is the reported regeneration of fertile *Arabidopsis*

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plants from immature cotyledons in the absence of 2,4-D
(Patton and Meinke, 1988). In addition, protocols have
been described for the regeneration of fertile
Arabidopsis plants from leaf explants, also in the
5   absence of 2,4-D (Lloyd et al., 1986; Van der Graaff and
Hooykas, 1996).

     As described in Example III, *Arabidopsis thaliana*
and *Brassica napus* belong to the same family, *Cruciferae*,
and therefore the plastid genomes share a high degree of
10   homology and are essentially identical (Palmer et al.,
1994). Accordingly, plastid transformation vectors and
expression cassettes developed for *Arabidopsis* can be
used for plastid transformation and expression of foreign
genes in *Brassica* species without modification.

15   The following definitions are provided to facilitate
an understanding of the present invention:

     Heteroplasmic: refers to the presence of a mixed
population of different plastid genomes within a single
plastid or in a population of plastids contained in plant
20   cells or tissues.

     Homoplasmic: refers to a pure population of plastid
genomes, either within a plastid or within a population
contained in plant cells and tissues. Homoplasmic
plastids, cells or tissues are genetically stable because
25   they contain only one type of plastid genome. Hence,
they remain homoplasmic even after the selection pressure
has been removed, and selfed progeny are also
homoplasmic. For purposes of the present invention,
heteroplasmic populations of genomes that are
30   functionally homoplasmic (i.e., contain only minor
populations of wild-type DNA or transformed genomes with
sequence variations) may be referred to herein as
"functionally homoplasmic" or "substantially
homoplasmic." These types of cells or tissues can be
35   readily purified to homoplasmy by continued selection on
the non-lethal selection medium. Most seed progeny of
such plants are homoplasmic in the absence of selection

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pressure, due to random sorting of plastid genomes.

Plastome: the genome of a plastid.

Transplastome: a transformed plastid genome.

Transformation of plastids: stable integration of transforming DNA into the plastid genome that is transmitted to the seed progeny of plants containing the transformed plastids.

Selectable marker: the term "selectable marker" refers to a phenotype that identifies a successfully transformed organelle, cell or tissue, when a gene or allele encoding the selectable marker is included in the foreign DNA used for transformation.

Transforming DNA: refers to homologous DNA, or heterologous DNA flanked by homologous DNA, which when introduced into plastids replaces part of the plastid genome by homologous recombination.

Targeting segment: refers to those homogologous flanking regions which facilitate homologous recombination between foreign DNA and the plastid genome.

Translationally fused: refers to two coding DNA segments within a construct derived from different sources spliced together in a construct such that a chimeric protein is expressed.

High auxin culture medium: plant tissue culture medium which contains auxin only, or a combination of high concentrations of auxin and very low concentrations of cytokinins. The response of a plant cell to an auxin is specific for a given taxonomic group. When different auxins are applied in combination, their effects may not be additive Furthermore, the tissue response to auxin may be modified by cytokinins. Accordingly, the type and concentration of auxin used should be determined empirically for the species to be transformed. A preferred example of a high auxin medium for use in the present invention is C1 medium, containing 1mg/ml of the auxin 1-napthtaleneacetic acid (NAA) and a low concentration (0.2 mg/ml) of the cytokinin 6-

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benzylaminopurine (BAP). Other auxins, such as indole-3-acetic acid (IAA) and dichloro-phenoxyacetic acid (2,4-D) may also be used to stimulate uniform cell division.

5   **High cytokinin culture medium:** like high auxin media, the response of plant cells to high cytokinin media is taxonomic group specific. An example of a preferred high cytokinin medium for use in the present invention is C medium, containing 1 mg/L of BAP, 2 mg/l of 2iP, (6-(gamma,gamma-Dimethylallyamino)purine or IPA,

10   N6-(Isopentenyl)adenine) and a low concentration of the auxin NAA (0.1 mg/L). Other cytokinins which may be used is 6-Furfurylaminopurine (KIN or kinetin).

The detailed description provided in the following examples relates to preferred methods for

15   making and using the DNA constructs of the present invention and for practicing the methods of the invention. Any molecular cloning and recombinant DNA techniques not specifically described are carried out by standard methods, as generally set forth, for example in

20   Sambrook et al., "DNA Cloning, A Laboratory Manual," Cold Spring Harbor Laboratory, 1989 or Ausubel et al. eds. in "Current Protocols in Molecular Biology", John Wiley and Sons, 1995.

The following examples are provided to more

25   fully describe the instant invention. They are not intended to limit the scope of the invention in any way.

EXAMPLE I

Plastid Transformation in Arabidopsis
30   #### Leaves by Selection for Spectinomycin Resistance

The following materials and protocols enable the practice of the methods of Example I. A schematic diagram of the methods utilized is provided in Figure 2.

35

Plant material

As the recipient for transformation, the *Arabidopsis* ecotype RLD was used. This ecotype has been

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reported to regenerate readily in culture (Marton and
Browse, 1991).

5   Construction of vector pGS31A
         The Arabidopsis plastid transformation vector pGS31A
      is shown in Figure 1. The immediate progenitor of pGS31A
      is plasmid pGS7, a pBluescript KS(+) phagemid vector
      (Stratagene) derivative. Plasmid pGS7 carries a 2-kb
10   HindIII-EcoRI Arabidopsis ptDNA fragment containing the
      5'-end of the 16S rRNA gene, trnV and part of the rps12/7
      operon. During construction of the pGS7 plasmid the
      HindIII site has been removed by digestion with HindIII
      (site in 16SrDNA) and KpnI (in vector, treated with the
15   T4 DNA polymerse to remove the single-stranded overhangs)
      and ligating the blunt ends. Vector pGS31A carries the
      selectable spectinomycin resistance gene,
      (Prrn::aadA::TpsbA) present in plasmid pZS197 (Svab and
      Maliga, 1993). The aadA coding region is transcribed from
20   a synthetic promoter consisting of the promoter of the
      tobacco rRNA operon fused with a synthetic ribosome
      binding site (Prrn). The aadA mRNA is stabilized by
      transcriptionally fusing sequences downstream of the
      coding region with the 3'-untranslated region of the
25   tobacco plastid psbA gene (TpsbA). The gene in pGS31A
      derives from a modified progenitor of pZS197 in which the
      XbaI site between aadA and TpsbA was removed by blunting.
      Plasmid pGS31A was obtained by excising the chimeric aadA
      gene with Ecl136II (an isochisomer of SacI, yields blunt
30   ends) and BspHI (single-stranded overhang filled in to
      obtain blunt ends) for ligation into the unique HincII
      site of plasmid pGS7 between trnV and the rps12/7 operon.

      Tissue culture media
35   The tissue culture protocols were adopted from
      Marton and Browse (1991) and Czako et al. (1993). The
      Arabidopsis tissue culture media (ARM) are derivatives of

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the Murashige & Skoog (1962) MS medium. ARM medium: MS
salts, 3% sucrose, 0.8% TC agar, 2 ml/L of the vitamin
solution (100 mg myo-inositol, 5 mg vitamin B1, 0.5 mg
vitamin B6, 0.5 mg nicotinic acid, 1 mg glycine and 0.05

5   mg biotin per ml). ARMI medium: ARM medium containing 3
mg indoleacetic acid (IAA), 0.15 mg 2,4-
dichlorophenoxyacetic acid (2,4-D), 0.6 mg benzyladenine
(BA) and 0.3 mg isopentenyladenine (IPA) per liter.
ARMIIr medium: ARM medium supplemented with 0.2 mg/L

10   naphthaleneacetic acid (NAA) and 0.4 mg/L IPA.
Arabidopsis shoot induction (ASI-N1B1) medium: ARM medium
supplemented with 1 mg/L NAA and 1 mg/L BAP. The
Arabidopsis shoots were rooted on ARM medium. *Arabidopsis*
seed culture (ARM5) medium: ARM medium supplemented with

15   5% sucrose. The stocks of plant hormones were filter
sterilized, and added to media cooled to 45 °C after
autoclaving.
        Selective media contained 500 mg/L
spectinomycin HCl and/or streptomycin sulfate. The

20   antibiotics (filter sterilized) were added to media
cooled to 45 °C after autoclaving.

Cultivation of *Arabidopsis* plants in sterile culture

25   For surface sterilization, seeds (25 mg) were
treated with 1 ml of commercial bleach (5.25% sodium
hypochlorite) in an Eppendorf tube for 5-7 minutes with
occasional vortexing. The seeds along with the bleach
were poured into a 15 ml conical centrifuge tube

30   containing 10 ml 90% ethanol and incubated for 5-7
minutes. The ethanol-bleach mix was decanted, and the
seeds were washed 4 times with 10 ml autoclaved deionized
water and finally resuspended in sterile water
(approximately 150 seeds/ml). The resulting seed

35   suspension (2 ml) was poured into 10 cm deep (10 mm high)
petri dishes containing 50 ml ARM5 medium. The seeds were
spread evenly by swirling the suspension. The water was

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then removed from the plates by pipetting. The seeds
germinated after a 10-15 day incubation at 24°C during
which the plates were illuminated for 8 hours using cool-
white fluorescent tubes (2000 lux).

5   To grow plants with larger leaves, seedlings
were individually transferred to ARM5 plates (10 plants
per 10 cm petri dish) and illuminated for 8 hours with
cool-white fluorescent bulbs (lux; 21°C day and 18°C
night temperature). The thick, dark green leaves, 1 cm
10   to 2 cm in size, were harvested for bombardment after 5-6
weeks.

Transformation and selection of spectinomycin resistant
lines

15   Leaves (approximately 1.5 to 30 mm in length)
for plastid transformation were harvested from
aseptically grown plants. To cover a circular area 4 to
5 cm in diameter, 12 to 18 leaves were placed on agar-
solidified ARMI medium. The pGS31A vector DNA was
20   introduced into leaf chloroplasts by the biolistic
process, on the surface of microscopic (1 μm) tungsten
particles using a helium-driven PDS1000 biolistic gun.
Fresh leaves were bombarded at 450 psi (target placed at
9 cm from rupture disk; position No. 3 from top in the
25   biolistic gun). Leaves cultured for 4 days on ARMI
medium were bombarded at 1100 psi (target placed at 12 cm
from rupture disk; position No. 4 from top in the
biolistic gun).

             Leaf bombardment was performed in ARMI medium.
30   Following bombardment, the leaves were incubated for two
additional days in ARMI medium. After this time period,
the leaves were stamped with a stack of razor blades to
create a series of parallel incisions 1 mm apart. It has
been observed previously that mechanical wounding is
35   essential to induce uniform callus formation in the leaf
blades. The stamped leaves were transferred onto the
same medium (ARMI) containing spectinomycin (500 mg/ml)

to facilitate preferential replication of plastids
containing transformed ptDNA copies. The ARMI medium
induces division of the leaf cells and formation of
colorless, embryogenic callus. After 7-10 day selection
on ARMI medium, spectinomycin selection was continued on
the ARMIIr medium which normally induces greening. Since
spectinomycin prevents greening of wild-type cells, only
spectinomcyin-resistant cells formed green calli. Visible
green cell clusters on the selective ARMIIr medium
appeared within 21 to 70 days.

In 201 bombarded samples 19 spectinomcyin-
resistant lines were obtained. Plant regeneration was
attempted in 14 spectinomycin-resistant lines, and
succeeded in 10 of them. Shoots from the green calli
regenerated on the ASI-N1B1 medium, and were rooted on
ARM medium.

Table 1 sets forth the recovery of spectinomycin
resistant cell lines after biolistic delivery of plasmid
pGS31A.

Table 1. Recovery of spectinomcyin resistant lines after bombardment
of *A. thaliana* with plasmid pGS31A

DNA[1]	Number of Samples	psi[2]	Number of Spc[r] Plant	Transgenic	pt Nucleus	Spont. mutant.
N/A	100		1	0		1
pGS31A	40	1100	8	6	1	7	0
pSG31A	151	450	11	8	1	10	0

[1]The control plates were not bombarded.
[2]psi=pounds per square inch, the value of repture disk.

Southern hybridizaton analysis of total
cellular DNA to verify plastid transformation

Spectinomycin resistance may be due to expression
of *aadA* in plastids (Svab and Maliga, 1993), expression
of *aadA* in the nucleus (Svab et al., 1990b), or
spontaneous mutation (Fromm et al., 1987; Svab and
Maliga, 1991).   Southern hybridization was performed to
indentify plastid tranformants in the spectinomycin

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resistant lines isolated. Total cellular DNA was
isolated according to Mettler (1987). Restriction enzyme-
digested DNA was electrophoresed in 0.7 % agarose gels
and transferred to nylon membrane (Amersham) using the
5 PosiBlot transfer apparatus (Stratagene). Blots were
probed by using Rapid Hybridization Buffer (Amersham)
with ^{32}P labeled probes generated by random priming
(Boehringer-Mannheim). When using the targeting ptDNA as
a probe, in lines At-pGS31A-2 and At-pGS31A-16, the 3.82-
10 kb transgenic fragment was the only fragment detected
indicating that the wild-type ptDNA copies have been
selectively diluted out during cell divisions on the
selective medium. The same transgenic fragment also
hybridized with the *aadA* probe (Figure 1C).
15   Among the 19 spectinomcyin resistant lines 17
nuclear transformants were identified by a wild-type
fragment on Southern blots when hybridizing with the
targeting ptDNA probe. Note that the Southern blots used
were optimized for the high-copy (10,000 per cell) leaf
20 ptDNA and will not give a signal with a few nuclear *aadA*
copies.
          Spontaneous mutants are expected to have wild-
type ptDNA targeting fragment on Southern blots and no
PCR-amplifiable *aadA* gene. In the sample of 19
25 spectinomycin resistant lines, no such spontaneous
mutant was found.

<u>PCR amplification of inserted *aadA* sequences</u>
30 DNA was amplified according to standard protocols (1 min
at 92 °C, 1.5 min at 58 °C, 1.5 min at 72 °C, 30 cycles).
Spectinomycin resistance being to result of aadA
expression may be verified by PCR amplification of an 407
nucleotide internal segment using the following primers:
35 5'-GCTTGATGAAACAACGCGG-3'
    5'-CCAAGCGATCTTCTTCTTGTCCAAG-3'

Transplastomic Arabidopsis Plants

        While the transplastomic *Arabidopsis* plants all
flowered, none of them set seed after selfing, or after
fertilization with pollen from wild-type plants. Included
among these were 98 plants regenerated from the two lines
in which spectinomcyin resistance was due to plastid
transformation, and 66 plants regenerated from 12 lines
in which spectinomcyin resistance was due to expression
of *aadA* in the nuclear genome.

Conclusions and Implications

        An important agricultural breakthrough, plastid
transformation in the model species *Arabidopsis thaliana*
is described in the instant invention. Based on the
foregoing results, it has been found that a chimeric *aadA*
gene, when inserted in the *Arabidopsis* ptDNA targeting
cassette, was suitable to recover plastid transformants
following biolistic delivery of the transforming DNA.
However, the number of *Arabidopsis* plastid transformants
was significantly lower, about one in 100, than
anticipated based on the transformation of tobacco
plastids which yields on average one transformant per
bombarded sample (Svab and Maliga, 1993; Zoubenko et al.,
1994). There may be multiple reasons for the relatively
low transformation efficiency. Inherent species-specific
differences, such as relatively inefficient homologous
recombination system in *Arabidopsis* chloroplasts could be
one obvious reason.

        In tobacco vector pZS197, *aadA* is flanked by
1.56-kb and 1.29-kb of ptDNA, and yields ⁻ 1 transformant
per bombardment (Svab et al., 1993). In plasmid pRB15,
also a tobacco vector, *aadA* is flanked by larger
targeting segments, 1.56-kb and 3.6-kb of ptDNA, and
yields approximately 5 plastid transformants per
bombardment (Bock and Maliga, 1995). In *Arabidopsis*
vector pGS31A *aadA* is flanked only by approximately 1-kb
plastid targeting sequence on both sides. Therefore, the

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efficiency of plastid transformation in *Arabidopsis* may
be significantly improved by increasing the size of the
targeting ptDNA fragment.

5   In contrast to tobacco, in which most of the
plants regenerated from leaves are fertile, it was
surprising to find that none of the 164 regenerated
Arabidopsis plants set seed. Lack of fertility, in part,
may be due to the extensive polyploidy of leaf tissue as
reported by Galbright et al., (1991) and Melaragno et al.

10   (1993).   An additional reason for lack of fertility may
be the prolonged exposure of the cultures to 2,4 D (Van
der Graaff and Hooykaas, 1996).

EXAMPLE II
Plastid Transformation in *Arabidopsis*
15   **Cotyledons by Selection for Kanamycin Resistance**

Plastid transformation has been obtained in
Arabidopsis thaliana by selection for spectinomycin
resistance in leaf cultures following bombardment with
DNA-coated tungsten particles, as set forth in Example I.

20   While plastid transformation has been successful, the
regenerated plants were not fertile. These obstacles
have been overcome by altering certain parameters of the
transformation protocol.

The protocol developed and set forth in this
25   Example has the following salient features: (1)
Cotyledons obtained by germinating mature seed are used
to advantage because of their ready availability, and the
ease by which large quantities of sterile cotyledons are
obtained from surface-sterilized seed.   (2) The protocol

30   has two distinct steps. The first step employing a high
auxin medium to induce uniform cell division throughout
the cotyledon (Stage I) and the second step including a
high cytokinin medium to induce plant regeneration
(Stages II and III). The protocol was designed to either

35   minimize exposure to medium containing 2,4-D during
tissue culture, or more preferably to eliminate such
exposure completely.   (3) Initial culturing of the

cotyledon cells at a high density, i.e., 500-200
cotyledons/ 20ml in liquid culture medium during the
first 8 days (Stage I, II) proved essential for obtaining
abundant plant regeneration later.

5   The protocol for plastid transformation in
Arabidopsis utilizing cotyledons as target tissue and
kanamycin-resistance as a selective marker was
implemented as follows. The chimeric kan gene derives
from plasmid pTNH7, a pUC118 derivative encoding
10   neomycin phosphotransferase (NPTII), an enzyme which
enzymatically inactivates the kanamycin antibiotic. The
same chimeric kan gene in a tobacco targeting
plasmid (plasmids pTNH32) was used for direct selection
of plastid transformants in tobacco (Carrer et al. 1993)
15   The construction of the kan gene was described
in more detail in this same reference. Plasmid pGS85A
was obtained by excising kan from pTNH7 as a SacI/PstI
fragment, blunting, and cloning the fragment into the
HincII site of plasmid pGS7 (Figure 4). The kan gene in
20   pGS85A, as aadA in plasmid pGS31A, is expressed in a
Prrn/TpsbA cassette. However, the five N-terminal amino
acids of the highly-expressed rbcL coding region were
translationally fused with the neomycin
phosphotransferase N-terminus. This translatinal fusion
25   in tobacco lead to the accumulation of NPTII at 10X
higher levels than from identical constructs without the
rbcL N-terminal segment. The DNA sequence of pGS85A,
including that of the chimeric kan gene, is set forth
herein.

30   Initially, seed-set was tested in plants
regenerated via the tissue culture protocol. Selection
of kanamycin resistant clones after bombardment with
DNA-coated tungsten was subsequently assessed. These
improvements to the method are suitable for the
35   generation of fertile, transformed *Arabidopsis* plants.
The following material and protocols were utilized in
practicing the methods of this Example II.

<u>Seed germination</u>

        Seeds of *Arabidopsis thaliana* ecotype RLD are
surface sterilized using commercial bleach (5 % sodium
hypochlorite) for 5 minutes followed by a subsequent 5
5   minute treatment with 95% ethanol. A drop of Triton
X-100 was added to the bleach to wet the surface of the
seeds during the sterilization period. After
sterilization, seeds were washed 5-6 times with sterile
deionized water. Seeds were germinated on GM medium in
10 cm Petri dishes. See Table 2. The Petri dishes were
incubated for 8 to 9 days in a Percival growth chamber at
23°C under continous light.

Table 2.**Composition of seed germination (GM) medium.**

Medium	Concentration (mg/L)
MS basal salts	0.5 X
myo-inositol	100
Thiamine	0.1
Pyridoxine	0.5
Nicotinic acid	0.5
Glycine	2.0
Sucrose	30 g/L
pH	5.8

Reference: van der Graaff and Hooykaas, 1996. .

25

<u>Tissue culture media and culture conditions</u>

        Compositions of the tissue culture media used for
Stages I, II and III of the selection protocol are listed
30 in Tables 2 and 3. Stage I and Stage II liquid cultures
were established by aseptically transferring at least 50
to 2000 cotyledons to a Petri dish (100 mm x 20 mm), each
dish containing approximately 20 ml of medium. The Petri
dishes were incubated at 23°C on a New Brunswick G10
35 gyrotory shaker at 60 rpm and illuminated for 16 hours
with cool fluorescent light. In the Stage III protocol,
cotyledons were incubated on agar-solidified (0.8 % TC
agar, JRH Biosciences) media at approximately 25-30
cotyledons per Petri dish (100 mm x 20 mm) in 50 ml of
40 media. The cultures were illuminated as described for
Stages I and II.

        Regenerated plants were directly transferred to

GM medium in Magenta boxes with vented lids for gas
exchange. Plants in the Magenta boxes were incubated in
the culture room at 23°C, and illuminated for 16 hours
with cool fluorescent light. The plants flowered and set
5   seeds in the boxes.

The methods described for Example I were modified
to generate fertile *Arabidopsis* plants having transformed
plastid genomes.   Three distinct tissue culture stages
were employed to obtain plastid transformation. Stage I:
10   liquid culture, in high auxin medium to stimulate uniform
cell division. Stage II: liquid culture, in high
cytokinin medium to induce plant regeneration from the
transformed cells. Stage III: culture on agar-solidified
medium, containing high levels of cytokinins also to
15   induce plant regeneration.

A schematic diagram of the strategy used to
identify the best protocol for obtaining fertile plastid
transformants is outlined in Figure 3. To induce uniform
cell division in liquid culture, four media, C1 (van der
20   Graaff and Hooykaas, 1996), ARM I (Marton and Browse,
1991), R3 and PG1 (Feldmann and Marks, 1986; reported to
induce callus and/or somatic embryogenesis in
Arabidopsis) were utilized. Stage I treatment was kept
short (2 days) to adopt to the usual timing of
25   transferring the explants to a selective medium after
bombardment, and to minimize the adverse effect of 2-4-D,
if used at all.   The composition of the Stage I tissue
culture media utilized is set forth in Table 3 below.

Table 3: Composition of Stage I tissue culture media*.

Media	ARM I	C1	R3	PG1
Basal salts	MS	MS	MS	MS
Vitamins	ARM I	B5	MS	B5
2,4-D	0.15	–	0.5	2.2
BAP	0.6	0.2	–	–
IAA	3.0	–	5.0	–
IPA	0.3	–	–	–
NAA	–	1.0	–	–
KIN	–	–	0.3	0.05
Sucrose	30g	30g	30g	30g
pH	5.8	5.8	5.8	5.8

*All components are in mg/L. References: ARM1, Marton and Browse,
1991; C1, van der Graaff and Hooykaas, 1996; R3 and PG1, Feldmann and
Marks (1986).

20

For Stage II culture, only one medium (A; Table 4) was used. This medium was efficient for inducing plant regeneration from immature cotyledons (Patton and Meinke, 1988). The cotyledons at Stage II were kept for a total of 6 additional days at high density in liquid culture.

For Stage III culture, the cotyledons were transferred to four types of agar-solidified regeneration media. These include the A medium developed for plant regeneration from immature embryos (Patton and Meinke, 1988); the B medium developed for plant regeneration from root explants (ARMII; Marton and Browse, 1991); the C medium that designed herein; and the D medium which is an embryo-induction medium for roots (ARMI; Marton and Browse, 1991) and leaf explants (Example I).

Table 4:Stage II and Stage III plant regeneration media.

Media	A medium*	B medium*	C medium*	D medium*
Basal salts	MS	MS	MS	MS
Vitamins	B5	B5	B5	B5
NAA	0.1	-	0.1	-
IAA	-	0.1	-	3.0
BAP	1.0	-	1.0	0.6
2iP	-	4.0	2.0	0.3
2,4-D	-	-	-	0.15
Sucrose	30 g	30 g	30 g	30 g
Agar (TC)	7 g	7 g	7 g	7 g
pH	5.8	5.8	5.8	5.8

*All components are in mg/L. A medium is based on Patton and Meinke, 1988; B medium is the same as ARMII in Marton and Bowse, 1991; C medium developed herein, based on A and D media; D medium is the same as ARMI embryo-induction medium in Marton and Browse, 1991.

Plant regeneration and Testing of Fertility

Cotyledons remained green and slightly expanded in size during the first 2 days of culture at Stage I in all four media. After 2 days in callus/embryo induction medium, cotyledons for Stage II were transferred from all four media to the A liquid regeneration medium. Green callus started appearing after 3 days of culture in A medium and by the 7th day callus appeared all over the cotyledons. At this stage cultures were transferred to

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the semi-solid media of Stage III which promotes
embryo/shoot growth. Calli derived from media 1 (ARM1)
and 2 (C1) were green. Development of plantlets from
these explants could be seen by 21 days of culture.
5   Callus derived from media 3 (R3) and 4 (PG1) was also
green but very compact. This is probably due to the high
concentration of 2,4-D in the Stage I media. A few
plantlets in these cultures appeared only after 30 days.
Plants from all cultures were transferred to hormone free
10   GM medium as soon as they were 5-10 mm in size.
        The protocols diagrammed in Figure 3 were
evaluated at two levels: uniform induction of cell
division and shoot regeneration from the cotyledons; and
by production of viable seed on the regenerated plants.
15   The results are summarized in Table 5. Based on the first
criterion, the best combination was 2AC, that is C1
medium at Stage I and C medium at Stage III, these
treatments resulted in prolific shoot regeneration which
was observed on each of the explants. The second best
20   combination was 1AC (35 out of 40 explants regenerating
shoots), with ARM1 at Stage I and medium C at Stage III.
Combinations with media 3 and 4 at Stage I performed very
poorly, with only a very small fraction of cotyledons
forming shoots.
25   As to formation of viable seed, with one
exception each of the regenerated plants produced viable
seed. See Table 5. Most importantly, no adverse effect
on fertility was found in the two combinations (2AC and
1AC) in which shoot regeneration is prolific.
30

35

Table 5

Seed-set in Magenta boxes on Arabidopsis thaliana RLD plants regenerated via plastid transformation protocols schematically shown in Figure 3.

Media	Number of cotyledons cultured	Number of cotyledons with shoots	Number of plants in boxes	Number of with viable seed
1AA	40	20	8	8
1AB	40	25	8	8
1AC	40	35	8	8
1AD	40	–	–	–
2AA	40	25	12	12
2AB	40	22	8	7
2AC	40	40	16	16
2AD	40	2	–	–
3AA	40	12	4	4
3AB	40	6	4	4
3AC	40	20	–	–
3AD	40	1	–	–
4AA	40	1	–	–
4AB	40	1	–	–
4AC	40	4	4	4
4AD	40	1	–	1

Selection of plastid transformants by kanamycin resistance

Expression of kan encoding neomycin phosphotrasferase (NPTII) confers resistance to kanamycin when introduced into the *Arabidopsis* nucleus. Engineered forms of kan have been extensively used to obtain nuclear transformants in *Arabidopsis*, see Valvekens et al., 1988. and *Brassica*, see Radke, et al. 1992. The kan gene has been converted into a plastid marker for the selection of plastid transformants in tobacco (Carrer et al., 1993). As set forth in Example I, *Arabidopsis* plastid transformants have been obtained by selection for spectinomycin resistance conferred by aadA in the tobacco Prrn/TpsbA cassette. Prrn is a promoter derived from the plastid rRNA operon and TpsbA contains the plastid psbA gene 3' untranslated region required for the stabilization of chimeric plastid mRNAs (Svab and Maliga, 1993). A kanamycin resistance marker gene suitable for the selection of plastid transformants may be obtained by expressing kan in the Prrn/TpsbA cassette. A suitable kanamycin resistance plastid transformation vector from

Arabidopsis and *Brassica* is the pGS85A vector which
carries the chimeric kanamycin gene from plasmid pTNH32
(Carrer et al. 1993). The insertion site in pGS85A is
the Hinc II site in the trnV/rps12/7 intergenic region.

5   However, other intergenic regions in the plastid genome
may be targeted as in providing the introduced transgene
does not interfere with the expression of the flanking
plastid genes.

      Plastid transformation may be carried out

10   following the 1AC or 2AC tissue culture protocols
outlined above. To prepare a suitable target tissue for
transformation, cotyledons from 8-9 day old seedlings are
cut from seedlings in liquid ARM1 and C1 media and
cultured for two days as dictated by the 1AC and 2AC

15   protocols (Figure 3). After two days the cotyledons are
transferred to filter paper (Whatman No. 4) on
agar-solidified non-selective medium of identical
composition. Approximately 50 to 70 cotyledons are
required to cover a 3 cm^2 area. The cotyledons are then

20   bombarded with plasmid pGS85A, a kanamycin resistance,
transforming *Arabidopsis* vector. Plasmid preparation,
coating of tungsten particles and bombardment should be
carried out as described for tobacco (Maliga, 1995). For
phenotypic expression, the cotyledons may be left in the

25   same plates for two days. Subsequently, the cotyledons
may be transferred to a selective liquid A medium
containing 50 mg/L kanamycin sulfate and cultured for an
additional seven days. After 7 days, cotyledons are
transferred to a selective, agar-solidified C medium

30   containing 50 mg/L kanamycin. In an alternative
embodiment, selection may be carried out initially using
kanamycin at 25 mg/ml. At later stages of culture, the
kanamycin concentration is increased to 50 mg/ml.
Callus growth from the transformed cells on the selective

35   medium may be observed as early as one week. However,
additional kanamycin-resistant clones may appear for
several more weeks. Some of these are plastid

transformants, while others acquire resistance to
kanamycin due to the expression of the plastid kan gene
in the nucleus (Carrer et al., 1993). The two classes of
kanamycin-resistant clones can be readily distinguished

5   DNA gel blot analysis and PCR analysis (as described in
Example I). DNA was amplified according to standard
protocols (1 min at 92 °C, 1.5 min at 58 °C, 1.5 min at
72 °C, 30 cycles). Kanamycin resistance occurs as the
result of neomycin phosphotransferase gene expression

10 which may be verified by PCR amplification of a 548
nucleotide internal segment using the following primers:
5'-CCGACCTGTCCGGTGCCC-3'
5'-CACGACGAGATCCTCGCCG-3'.

15

EXAMPLE III
Plastid Transformation in Brassica napus Leaves by
selection for Resistance to spectinomycin and kanamycin

20
        Given their essentially identical genomes,
plastid transformation vectors and expression cassettes
developed for *Arabidopsis* can be used to advantage for
plastid transformation and expression of foreign genes in

25 *Brassica* species without modification.
        Certain plastid expression signals derived from
evolutionarily distant species function in *Arabidopsis*
and *Brassica* plastids. This observation is supported by
the results described in Example I demonstrating that the

30 tobacco Prrn/TpsbA cassette can be used for expressing
the selectable spectinomycin resistance gene (aadA) in
Arabidopsis plastids. However, not every tobacco
Lxpression signal functions properly in *Arabidopsis*.
Studies with a vector identical to PGS31A, except that

35 the termination signal TpsbA, has been replaced with
signal Trps16 has a dramatic effect on obtaining plastid
transformants. This plasmid gene was obtained by
inserting the Prrn/Trps16 cassette into targeting site in
the pGS7 vector. See figure 4. Zero plastid

transformants were obtained out of 416 samples bombarded
with this plasmid. As mentioned above, when a
Prrn/TpsbA cassette (cassettes described in Staub and
Maliga, Plant Journal 6:547-553, 1994 and Svab and
5   Maliga, 1993, the subject matter of which is incorporated
herein by reference) was utilitized to transform
Arabidopsis leaves, plastid transformants were obtained,
2 out of 210 samples bombarded.

            Due to their taxonomic relatedness, *Arabidopsis*
10   and *Brassica* species respond similarly in tissue culture
to plant hormones or to antibiotics. As a result, plant
regeneration from cultured cells and selection of
transgenic lines by antibiotic resistance may be
accomplished by essentially the same protocol. Both
15   *Arabidopsis* and *Brassica* leaf or cotyledon explants
respond to 500 mg/L spectinomycin with prolific callus
growth in wild-type, non-transformed tissue on shoot
regeneration medium, such as medium C described in Table
6. This response differs significantly in tobacco leaf
20   tissue wherein exposure to 500 mg/ml of spectinomycin
results in a severe inhibition of callus proliferation on
shoot induction medium. Thus, tobacco plastid (and
nuclear gene) transformants can be readily regenerated on
a shoot induction medium containing spectinomycin at 500
25   mg/L (Svab and Maligam 1993). Unfortunately, rapid
callus proliferation on spectinomycin-containing C
shoot/embryo regeneration medium (see table 6) prevents
the recovery of *Arabidopsis* and *Brassica* plastid
transformants. Culture conditions must be improved to
30   suppress rapid callus growth to facilitate the recovery
of plastid transformants. Such conditions are outlined
in Example I. While selection was feasible and plastid
transformants were obtained using the methods of Example
I, the transplastomic plants generated were not fertile.
35   However, given the higher tolerance of *Brasssica* to 2,4 D
(Radke et al., 1992) the same protocol described in
example I may be adapted for use in *Brassica*.

The data presented in Example II indicate that kanamycin selection is compatible with the regeneration protocols described. Accordingly, kanamycin is the favored antibiotic for the selection of plastid

5   transformants in the *Cruciferae* taxonomic group.

Examples I and II disclose protocols for the regeneration of transgenic plants from *Arabidopsis* leaves and cotyledons. A protocol for the regeneration of transgenic plants in *Brassica* would involve a two-stage

10   protocol (application of two different media) for leaves, and a three-stage protocol (application of three different media) for cotyledons. The three-stage protocol described for use in the plastid transformation of *Arabidopsis* cotyledons in Example II is suitable for

15   use in Brassica. Accordingly only the methods for transforming Brassica leaf plastids in a two stage process will be described below .

Plastid transformation in Brassica utilizing leaves as target tissue and kanamycin resistance as the selective
20   **marker**

Brassica Stage I culture results in the uniform induction of cell division in leaves or cotyledons. The objective of Stage II is regeneration of transgenic

25   plants. A suitable Stage I medium for the induction of cell division would be the ARMI medium discussed in Examples I and II. Suitable Stage II regeneration media would be the B medium (ARMII in Marton and Browse, 1991), C medium (this study) and E medium (Pelletier et al.

30   1983) listed in Table 6.

Table 6 Stage II Brassica plant regeneration media*

Media	B medium	C medium	E medium
Basal salts	MS	MS	MS
Vitamins	B5	B5	B5
NAA	–	0.1	1.0
IAA	0.1	–	–
BAP	–	1.0	–
2iP	4.0	2.0	1.0
GA3	–	–	0.02
Sucrose	30 g	30 g	30 g
Agar (TC)	7 g	7 g	7 g
pH	5.8	5.8	5.8

*All components are in mg/L. B medium is the same as ARMII in Marton and Bowse, 1991; C medium is this study; E medium is the cruciferae

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regeneration medium of Pelletier et al. 1983.

For selection of plastid transformants, *Brassica*
napus cv. Westar seeds should be surface sterilized, and
germinated aseptically in Magenta boxes as described for
Arabidopsis in Example II. After three to four weeks,
the leaves are harvested, and directly placed a Whatman
filter paper placed on agar-solidified non-selective
Stage I medium. Following bombardment with DNA of the
appropriate plastid transformation vector carrying a
selectable kanamycin-resistance marker, as described in
Example II, the plates are incubated for two days in the
light (16 hours) at 25°C. After 2 days the leaves are
incised with a stack of sterile razor blades, and
transferred to the same Stage I medium supplemented with
50 mg/L of kanamycin sulfate. In an alternative
embodiment, selection may be carried out initially using
kanamycin at 25 mg/ml. At later stages of culture, the
kanamycin concentration is increased to 50 mg/ml.After
two weeks on the selective Stage I medium, the leaves are
transferred to one of the Stage II media for plant
regeneration. Kanamycin resistant clones are identified
by their rapid growth and shoot regeneration on the
selection medium. Kanamycin resistance may be due to
plastid transformation or integration of the kanamycin
marker gene into the nuclear genome. Plastid
transformation is verified by PCR and DNA gel blot
analysis in tissue samples taken from kanamycin-resistant
calli and regenerating shoots. The regenerated shoots
are then rooted and transferred to soil in the greenhouse
following standard protocols.

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234:464-466.

33.   Patton, D.A. and Meinke , D.W. (1988)
High-frequency plant regeneration from cultured
cotyledons of Arabidopsis thaliana. Plant Cell Reports
7:233-237.

31

34.   Van der Graaff, E. and Hooykaas, P.J.J. (1996)
Improvements in the transformation of Arabidopsis
thaliana. C24 leaf-discs by Agrobacterium tumefaciens .
5   Plant Cell Rep. 15: 572-577.

35.   Pelletier, G., Primard, C., Vedel, F., Chetrit,
P. Remy, R. Rousselle, R. and Renard M. (1983)
10   Intergeneric cytoplasmic hybridization in cruciferae by
protoplast fusion. Mol. Gen. Genet. 191:244-250.

36.   Radke, S. E., Turner, J. C., and Facciotti D. (1992)
15   Transformation and regereation of Brassica rapa using
Agrobacterium tumefaciens. Plant Cell Reports 11:499-505.

        While certain preferred embodiments of the
20   present invention have been described and specifically
exemplified above, it is not intended that the invention
be limited to such embodiments. Various modifications
may be made to the invention without departing from the
scope and spirit thereof as set forth in the following
25   claims.

What is claimed is:

     1.   A method for obtaining transplastomic plants, comprising:

     a)   culturing plant cells from said plant in the presence of an agent that stimulates uniform cell division;

     b)   transferring said plant cells to filter paper on agar-solidified medium;

     c) delivering to a plastid genome within said plant cells, a transforming DNA, said transforming DNA molecule having:

     i)   a plurality of targeting segments consisting of plastid DNA sequences from said plastid genome to be transformed, said targeting segments facilitating homologous recombination of said transforming DNA into said plastid genome;

     ii) 5' and 3' regulatory sequences derived from plastid DNA operably linked to a selectable marker gene disposed within said targeting segment, said regulatory sequences facilitating expression of the selectable marker gene and stability of mRNA encoded therefrom, said selectable marker gene conferring antibiotic resistance to said plant cells;

     iii) 5' and 3' regulatory sequences derived from plastid DNA operably linked to sequences encoding a foreign gene of interest thereby facilitating expression of the foreign gene of interest and stability of mRNA encoded therefrom; and

     iv)   at least one cloning site for insertion of said foreign gene of interest adjacent to said selectable marker gene, said insertion not interfering with said conferring of said selectable phenotype and function of flanking plastid genes;

     d)   transferring cells transformed as in step (c) to a culture medium at high density for a predetermined time period; said culture medium containing an agent that promotes continued uniform cell division and plant

regeneration;

        e)   transferring said cells treated as in step
(d) to an agar-solidified culture medium containing said
regeneration promoting agent and said antibiotic, said
5   transformed cells being rendered resistant to said
antibiotic by expression of said selectable marker gene;
and

        f) selecting for cells having transformed plastid
genomes and inducing plant regeneration therefrom.
10

        2.   A method as claimed in claim 1, wherein said
transforming DNA is delivered by a method selected from
the group consisting of biolistic bombardment of said
cells with DNA-coated particles, $CaPO_4$ mediated
15   transfection, eletroporation, and polyethylene glycol
mediated DNA uptake.

        3.   A method claimed as in claim 1, wherein said
plant cells are selected from the group consisting of
20   cotyledon cells, leaf cells, hypocotyls and root cells.

        4.   A method as claimed in claim 1, wherein said
selectable marker gene and said foreign gene of interest
constitute a monocistronic expression unit.
25

        5.   A method as claimed in claim 1, wherein said
selectable marker gene and said foreign gene of interest
constitute a polycistronic expression unit.

30   6.   A method as claimed in claim 1, wherein said
plastids are chloroplasts.

        7.   A method as claimed in claim 1, wherein said
antibiotic is selected from the group consisting of
35   kanamycin, spectinomycin, and streptomycin.

        8.   A method as claimed in claim 1, wherein said

34

agent promoting uniform cell division is selected from
the group consisting of NAA, IAA, and 2,4-D.

9.   A method as claimed in claim 1, wherein said
regeneration promoting agent is selected from the group
consisting of BAP, 21P IPA and KIN.

10.   A method as claimed in claim 1, wherein said
transforming DNA is cloned within vector pGS31A.

11.   A method as claimed in claim 1, wherein said
transforming DNA is cloned within vector pGS85A.

12.   A method as claimed in claim 1, wherein said
transforming DNA is cloned within vector PGS7.

13.   A method as claimed in claim 1, wherein said
plant is *Arabidopsis thaliana*.

14.   A method as claimed in claim 1, wherein said
plant is *Brassica napus*.
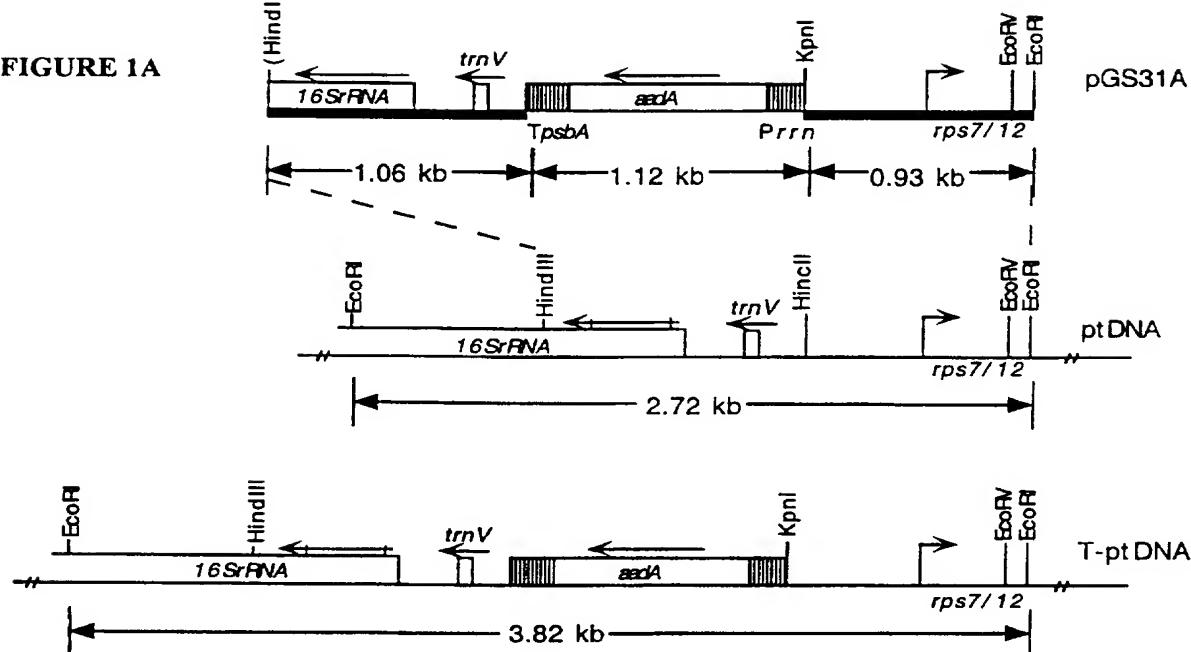
**FIGURE 1A**

FIGURE 1B
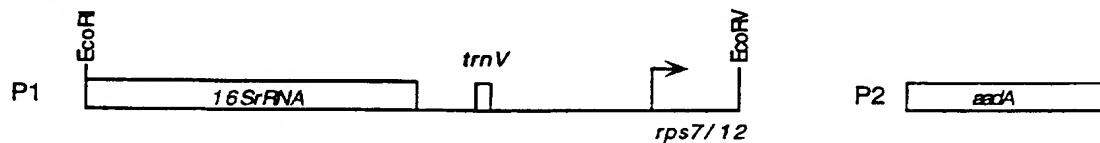
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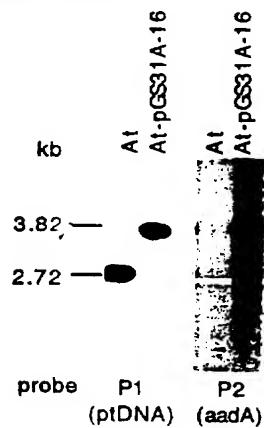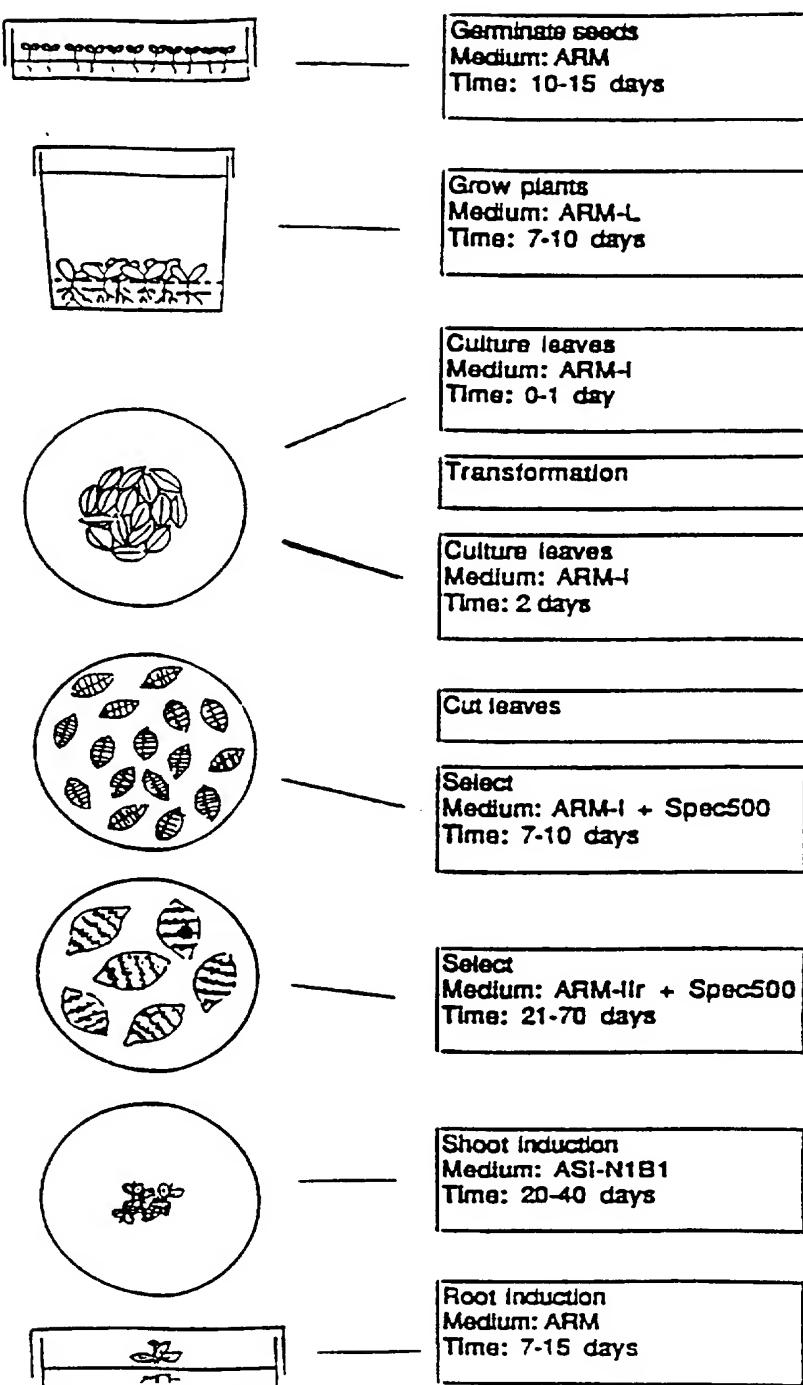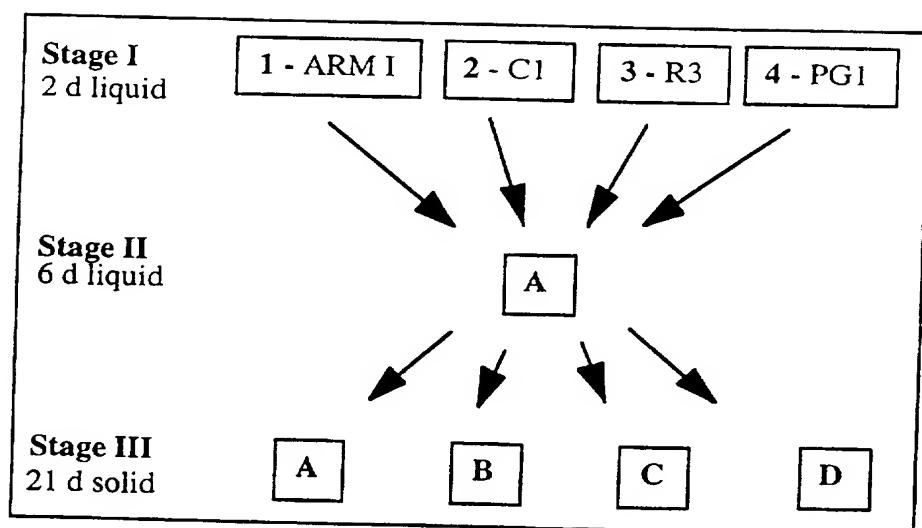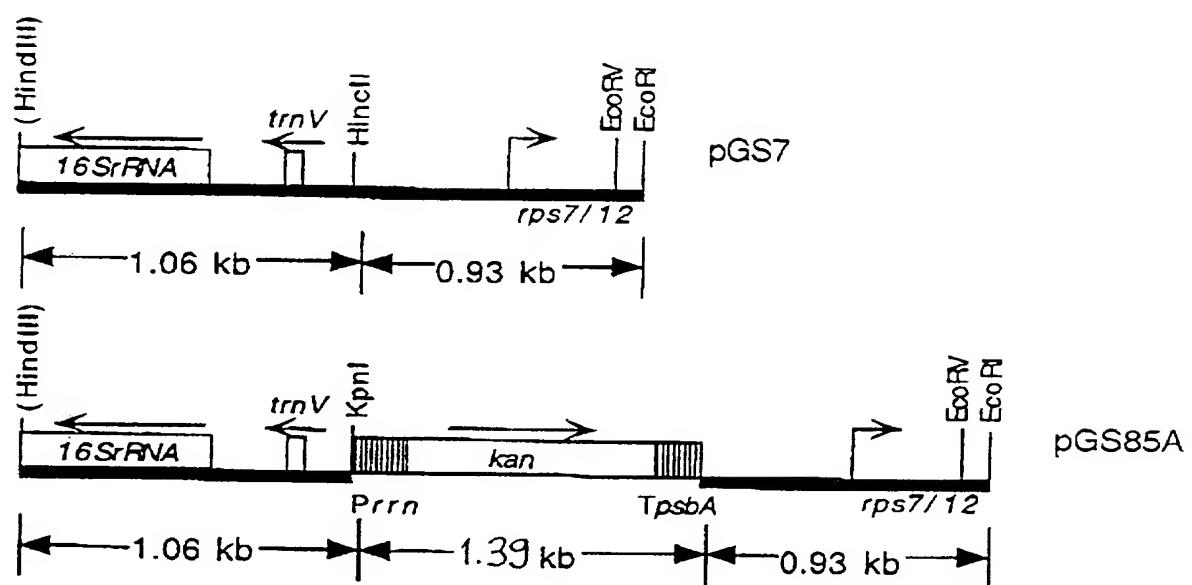
FIGURE 2

FIGURE 3

FIGURE 4

HindIII

```
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 101  TAACGCTTGC ATCCTCTGTA TTACCGCGGC TGCTGGCACA GAGTTAGCCG
 151  ATGCTTATTC CCCAGATACC GTCATTGCTT CTTCTCTGGG AAAAGAAGTT
 201  CAGGACCCGT AGGCCTTCTA CCTCCACGCG GCATTGCTCC GTCAGGCTTT
 251  CGCCCATTGC GGAAAATTCC CCACTGCTGC CTCCCGTAGG AGTCTGGGCC
 301  GTGTCTCAGT CCCAGTGTGG CTGATCATCC TCTCGGACCA GCTACTGATC
 351  ATCGCCTTGG TAAGCTATTG CCTCACCAAC TAGCTAATCA GACGCGAGCC
 401  CCTCCTCGGG CGGATTCCTC CTTTTGCTCC TCAGCTACGG GGTATTAGCA
 451  GCCGTTTCCA GCTGTTGTTC CCCTCCCAAG GGNAGGTTCT TACGCGTTAC
 501  TCACCNGTCC GCCACTGGAA ACACCACTTC CCGTCCGACT TGCATGTGTT
 551  AAGCATGCCG CCAGCGTTCA TCCTGAGCCA GGATCGAACT CTCCATGAGA
 601  TTCATAGTTG CATTACTTAT AGCTTCCTTC TTCGTAGACA AAGCTGATTC
 651  GGAATTGTCT TTCATTCCAA GTCATAACTT GTATCCATGC GCTTCATATT
 701  CGCATGGAGT TCGCTCCCAG AAATATAGCT ACCCCTACCC CCTCACGTCA
 751  ATCCCACGAG CCTCTTATCC ATTCTTATTC GATCACAGCG AGGGAGCAAG
 801  TCAAAATAGA AAAACTCACA TTCATTGGGT TTAGGGATAA TCAGGCTCGA
 851  ACTGATGACT TCCACCACGT CAAGGTGACA CTCTACCGCT GAGTTATATC
 901  CCTTCCCCCA TCAAGAAATA GAACTGACTA ATCCTAAGTC AAAGGGTCGA
 951  GAAACTCAAG GCCACTATTC TTGAACAACT TGGATTGGAG CCGGGCTTTC
1001  CTTTCGCACT TTATACGGGT ATGAAATGAA AATAATGGAA AAAGTTGGAT
1051  TCAATTGTCA ACTACTCCTA TCGGAAATAG GATTGACTAC GGATTCGAGC
1101  CATAGCACAT GGTTTCATAA AACCGTACGA TTCTCCCGAT CTAAATCAAG
1151  CCGGTTTTAC ATGAAGAAGA TTTGACTCGG CATGTTCTAT TCGATACGGG
1201  TAGGAGAAAC GGTATTCTTT TCTTAAACTT CAAAAAATAG AGAAATAAGA
1251  ACCAAGTCAA GATGATACGG ATTAATCCTT TATTCTTGCG CCAAAGATCT
1301  TCCTATTCCA AGGAACTGGA GTTACATCTC TTTTCCATTT CCATTCAAGA
1351  GTTCTTATGT GTTTCCACGC CCCTTTAAGA CCCGAAAAA TCGACAAATT
1401  CCCTTTTCTT AGGACCACAT GCGAGATAAC GAAAAAAAAA AAGAGAGAAT
1451  GGTAACCCCA CGATTAACTA TTTTATTTAT GAATTTCATA GTAATAGAAA
1501  TACATGTCCT ACCGAAACAG AATTTGTAAC TTGCTATCCT ATCATCTTGC
1551  CTAGCAGGCA AAGATTTCAC TCCGCGAAAA AGATGATTCA TTCGGATCAA
1601  CATGAAAGCC CAACTACATT GCCAGAATTT ATATATTGGA AAGAGGTTTA
1651  CCTCCTTGCT TCTATGGTAC AATCCTCTTC CCGCGGAGCC TCCTTTCTTC
1701  TCGGTCCGCA GAGACAAAAT GTAGGACTGG TGCCAACAGT TAATCACGGA
1751  AGAAAGGACT CACTGCGCCA AGATCACTAA CTAATCTAAT AGAATAGAAA
1801  ATCCTAATAT AATAGAAAAG AAAAGAACTG TCTTTTCTGA TACTTATGTA
1851  TACTTTCCCC GGTTCCGTTG CTACTGCGGS TTTACGCAAT TGATCGGATC
1901  ATCTAGATAT CCCTTCAACA CAACATAGGT CGTCGAAAGG ATCTCGGAGA
1951  CCCGCCAAAG CACGAAAGCC AGAATCTTTC AGAAAATGAA TTC
```

EcoRI

HincII

FIGURE 5

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blunt from pZS179, XbaI site filled in
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 51    CAAAAAGTC TATGTAAGTA AAATACTAGT AAATAAATAA AAAGAAAAA

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301    agataagcct gtctagcttc aagtatgacg ggctgatact gggccggcag

351    gcgctccatt gcccagtcgg cagcgacatc cttcggcgcg attttgccgg

401    ttactgcgct gtaccaaatg cgggacaacg taagcactac atttcgctca

451    tcgccagccc agtcgggcgg cgagttccat agcgttaagg tttcatttag

501    cgcctcaaat agatcctgtt caagaaccgg atcaaagagt cctccgccg

551    ctggacctac caaggcaacg ctatgttctc ttgcttttgt cagcaagata

601    gccagatcaa tgtcgatcgt ggctggctcg aagatacctg caagaatgtc

651    attgcgctgc cattctccaa attgcagttc gcgcttagct ggataacgcc

701    acggaatgat gtcgtcgtgc acaacaatgg tgacttctac agcgcggaga

751    atctcgctct ctccagggga agccgaagtt tccaaaaggt cgttgatcaa

801    agctcgccgc gttgtttcat caagccttac ggtcaccgta accagcaaat

851    caatatcact gtgtggcttc aggccgccat ccactgcgga gccgtacaaa

901    tgtacggcca gcaacgtcgg ttcgagatgg cgctcgatga cgccaactac

951    ctctgatagt tgagtcgata cttcggcgat caccgcttcT GCcatAAATC

1001   CCTCCCTACA ACTGTATCCA AGCGCTTCGT ATTCGCCCGG AGTTCGCTCC

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Figure 6
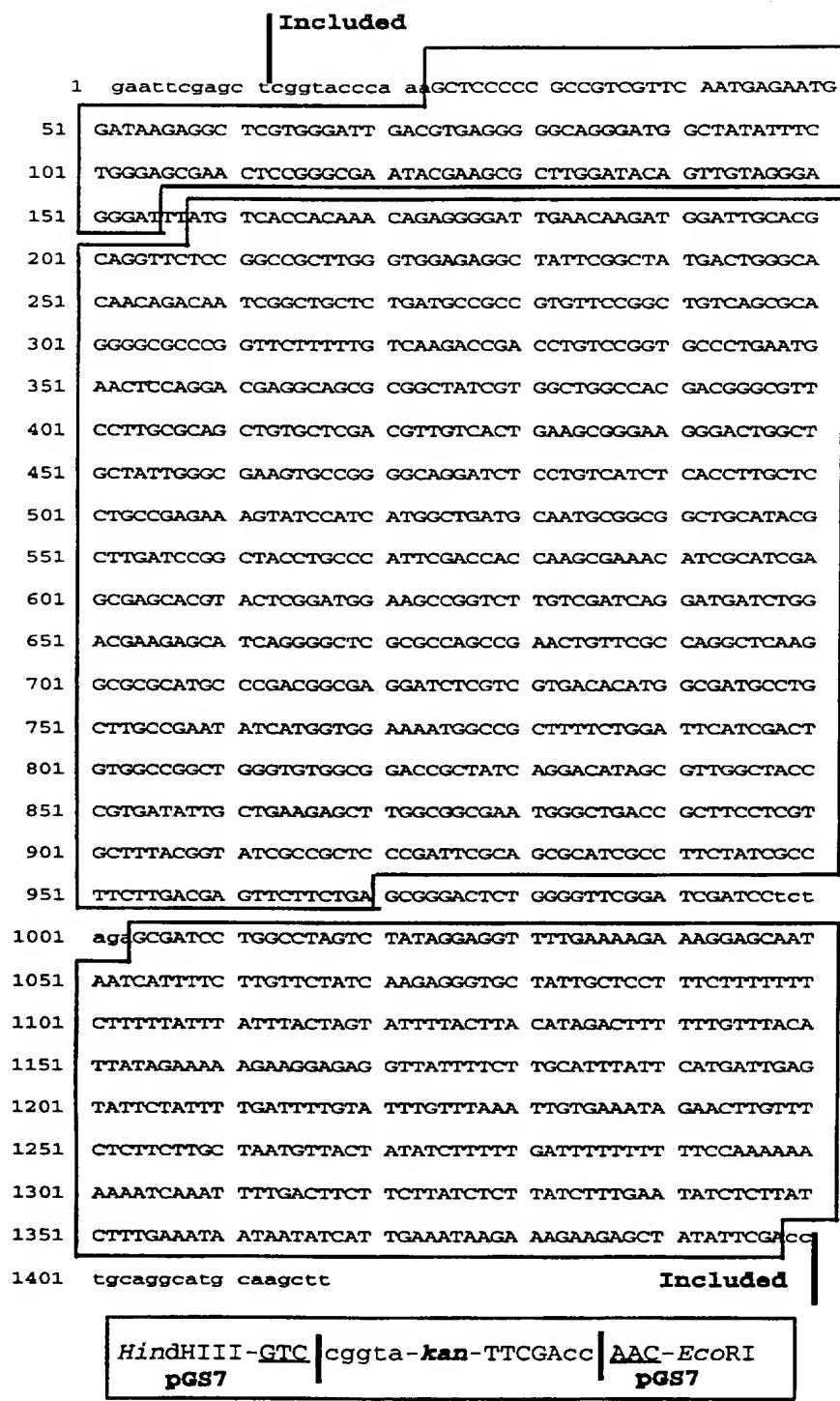
FIGURE 7

INTERNATIONAL SEARCH REPORT

A.　CLASSIFICATION OF SUBJECT MATTER

IPC(6)　:C12N 15/00, 15/63
US CL　:435/172.1, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B.　FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :　435/172.1, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.　DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,451,513 A (MALIGA et al.)　19 September 1995, columns 4-5 and 7-12.	1-14
Y, P	US 5,545,817 A (MCBRIDE et al.) 13 August 1996, columns 2-7.	1-14
Y, P	US 5,545,818 A (MCBRIDE et al.) 13 August 1996, columns 2-6.	1-14
Y, P	US (5,576,198) A (MCBRIDE et al.) 19 November 1996, columns 2-11.	1-14
Y	Koop et al. 'Plastid Transformation by Polyethylene Glycol Treatment of Protoplasts and Regeneration of Transplastomic Tobacco.' In: Gene Transfer to Plants. Edited by I. Potrykus et al. New York: Springer-Verlag, 1995, pages 75-82, see entire document.	1-14

| X | Further documents are listed in the continuation of Box C. | | | See patent family annex. |

* Special categories of cited documents:

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"E"　earlier document published on or after the international filing date

"L"　document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O"　document referring to an oral disclosure, use, exhibition or other means

"P"　document published prior to the international filing date but later than the priority date claimed

"T"　later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"　document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"　document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"　document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 APRIL 1997	0 1 JUL 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	THANDA WAI
Facsimile No.　(703) 305-3230	Telephone No.　(703) 308-0196

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, EPO, JPO, BIOSIS, EMBASE, AGRICOLA

search terms: ?plastid?, ?plastom?, transgen?, transplastom?, arabidopsis

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	STAUB et al. Long Regions of Homologous DNA Are Incorporated into the Tobacco Plastid Genome by Transformation. The Plant Cell. January 1992. Vol. 4, No. 1, pages 39-45, especially pages 39-42.	1-14
Y	STAUB et al. Marker Rescue from the Nicotiana tabacum Plastid Genome Using a Plastid/ Escherichia coli Shuttle Vector. Molecular General Genetics. 1995. Vol. 249, pages 37-42, especially pages 37-40.	1-14
Y	CARRER et al. Kanamycin Resistance as a Selectable Marker for Plastid Transformation in Tobacco. Molecular General Genetics. October 1993. Vol. 241, pages 49-56, especially pages 49-54.	1-14
Y	ZOUBENKO et al. Efficient Targeting of Foreign Genes into the Tobacco Plastid. Nucleic Acids Research. 25 September 1994. Vol. 22, No. 19, pages 3819-3824, especially pages 3819-3822.	1-14
Y	CHAUDHURI et al. Site-Specific Factor Involved in the Editing of the psbL mRNA in Tobacco Plastids. EMBO Journal. 1995. Vol. 14, No. 12, pages 2951-2957, especially pages 2951-2955.	1-14
Y	STAUB et al. Expression of a Chimeric uidA gene Indicates that Polycistronic mRNAs Are Efficiently Translated in Tobacco Plastids. The Plant Journal. 1995. Vol. 7, No. 5, pages 845-848, especially pages 845-847.	1-14